Add new Claims 78 and 79.

80

~~78.~~ (New) A method for treating a disease in a mammal, comprising administering an effective amount of nitrosated hemoglobin to the mammal, wherein the disease is selected from the group consisting of heart disease, brain disease, vascular disease, atherosclerosis, lung disease and inflammation.

81

~~79.~~ (New) A blood substitute comprising nitrosated hemoglobin.

REMARKS

A missing word has been added to the written description on page 77, line 19. It will be seen that the addition of "acetate" at this site agrees with the written description at page 31, line 35.

Claim 16 has been amended. For support of Claim 16 as amended, see page 18, line 15 to page 19, line 3.

Claims 67, 68, 70 and 71 have been amended to more accurately define the invention. Support for Claims 67 and 68 as amended can be found, for instance, on page 47, line 14 to page 48, line 13, on page 48, line 34 to page 49, line 8, on page 69, lines 21-28, and in Figure 1D. Support for Claims 70 and 71 as amended can be found on page 47, lines 1-5 and in Figure 1C, for example.

Claims 78 and 79 have been added, and are derived from Claims 21 and 29, respectively. For support for Claims 78 and 79, see page 4, lines 11-29; page 20, line 33 to page 21, line 13; page 24, lines 1-8; and page 31, line 9 to page 32, line 24.

Definitions of "Nitrosated Hemoglobin" and "Nitrated Hemoglobin"

A definition to be used in interpreting the claims reciting "nitrosated hemoglobin" appears on page 27, lines 9-20. It should be seen from this definition that S-nitrosohemoglobin, (SNO-hemoglobin), nitrosylhemoglobin (having NO bound to the heme Fe), and polynitrosated

hemoglobin are all within this definition. S-nitrosohemoglobin is also referred to as S-nitrosylhemoglobin, SNO-hemoglobin or SNO-Hb, and can be in the forms of SNO-Hb[Fe(II)] (also written as SNO-deoxyHb), SNO[Fe(II)]O_2 (also written as SNO-oxyHb), or SNO[Fe(III)] (also written as SNO-metHb or SNO-methemoglobin). Polynitrosated hemoglobin has NO adducts at additional sites (*e.g.*, O, N, or possibly C atoms) besides both sulfur atoms of the β93 cysteines, and therefore, can also be considered a form of S-nitrosohemoglobin. One of ordinary skill in the art would predict this to be the result of the order of the reactivity of the nucleophilic sites of the hemoglobin molecule, known from general chemical principles: S > O > N > C. Thus, a nitrosated hemoglobin has at a minimum, an NO group bound at a heme Fe or at a sulfur atom derived from a thiol group.

The term "nitrated hemoglobin" is not given any special definition in the written description. Thus, it will be understood, from the meaning of "nitrate" in chemistry, that "nitrated hemoglobin" is a species of hemoglobin having one or more nitrate groups. It will also be understood by one of ordinary skill in the art that agents facilitating nitrosations, nitrosylations or nitrations will result in the attachment of NO_x groups, depending on the attachment site and conditions. See the written description at page 26, line 30 to page 27, line 2.

Nitrosated Hemoglobins -- Teachings of the Prior Art and Teachings of the Subject Application

Nitrosylhemoglobin (NO bound at one or more heme Fe atoms) was known in the prior art. It was known that the affinity of NO for the heme Fe is extremely high and that the reaction in which nitrosylhemoglobin dissociates to NO and unbound hemoglobin has an extremely low rate, so low that the binding of NO to the Fe of hemoglobin can be considered essentially irreversible. For a useful comparison of the affinities of NO, CO and O_2 to the heme Fe, see Greenburg, A.G. and H.W. Kim, *Art. Cells, Blood Subs., and Immob. Biotech. 23*:271-276, 1995, especially fifth paragraph on page 272 (reference AX), wherein it is said that the affinity of NO for the heme Fe is 1,000 times that of CO and 200,000 times that of O_2. For k_{on} and k_{off} rates for NO in the reaction NO + hemoglobin ↔ nitrosylhemoglobin, see reference AR4, Kharitonov *et al.*, pp. 39-45 in *Methods in Nitric Oxide Research*, Feelisch and Stamler, eds., John Wiley and

Sons, Ltd., 1996, especially Table 2, page 41, from which it can be seen that the dissociation constant for NO from nitrosylhemoglobin is on the order of 10^{-12}. The other forms of nitrosated hemoglobins were unknown before the work described in this application and the priority applications.

This application describes the synthesis and physiological effects of new species of hemoglobin unknown in the prior art (S-nitrosohemoglobins), and shows that S-nitrosohemoglobin is an endogenous species of hemoglobin present at low concentrations as a normal constituent of hemoglobin in the red blood cells (see Example 8 in the written description, page 65, line 1 to page 66, line 10). The application also describes a previously unknown intramolecular reaction in which nitrosylhemoglobin is converted, through an intramolecular reaction that occurs at physiological conditions, to S-nitrosohemoglobin. Thus, at physiological conditions *in vivo*, nitrosylhemoglobin can be converted to S-nitrosohemoglobin, and therefore, can be useful in methods of therapy as a donor of NO. See, *e.g.*, Examples 18 and 19, on page 75, line 5 to page 76, line 6 of the written description. See also page 28, line 22 to page 30, line 33.

Additional Comments Regarding Cited Reference Stamler *et al.* (WO 93/09806)

The logic behind the experiments described in WO 93/09806 that one of ordinary skill in the art might take from the written description in WO 93/09806 is that because proteins contain thiol groups (at cysteine residues), S-nitrosylated proteins might be used as NO donors, just as some low molecular weight S-nitrosothiols were already known to be NO donors.

Several proteins which were readily available in purified form (tissue plasminogen activator, bovine serum albumin, cathepsin B, lipoprotein and immunoglobulin) were used as substrates in nitrosylation reactions. The reactions were apparently successful with these proteins, and yielded S-nitrosoproteins. Enzymatic activity and conformational integrity of these S-nitrosoproteins were not tested. However, proteins that have been treated with acidified nitrate in the method used in WO 93/09806 (excess $NaNO_2$ in 0.5 N HCl) are denatured.

An attempt was made (described in Example 19 of WO 93/09806) to produce a similar reaction using hemoglobin as a substrate. However, at that time, the choice to include hemoglobin as one of the proteins that could possibly act as a donor of NO when S-nitrosylated was somewhat illogical. It had been generally thought that nitric oxide reacted with hemoglobin in two major ways: 1) with the deoxyhemoglobin to form a stable nitrosyl (FeII) heme adduct (nitrosylhemoglobin); and 2) with oxyhemoglobin to form nitrate and methemoglobin -- a reaction that inactivates NO. These two reactions contributed to the idea that hemoglobin is a scavenger of NO (Wennmalm *et al., Br. J. Pharmacol. 106*:509-510, 1992; reference AU4). In both of these reactions, NO biological activity is lost. Thus, it was somewhat illogical to think that hemoglobin would act as a nitric oxide donor like low molecular weight S-nitrosothiols, when the prior art indicated that any NO in the vicinity of the heme Fe would be bound and trapped by the heme Fe and inactivated and/or eliminated by the other mechanism.

No nitrosohemoglobin was produced by any methods described in WO 93/09806. It was demonstrated by experimental evidence (Exhibits E1-E3; see also item 5 on page 3 of Declaration) described in the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the Patent Office on January 6, 1999, that no S-nitrosohemoglobin was produced in an attempt to reproduce the experimental conditions presented in Example 19 on pages 58-59 of WO 93/09806. Because no S-nitrosohemoglobin could be made by this method, it is most reasonable to conclude that NO adducts did not result at O, N, or C sites on hemoglobin by this method. The thiol groups of the hemoglobin molecule are more reactive nucleophilic sites than O, N or C sites. The product made by the method of Example 19 is dissociated subunits of hemoglobin, with the heme iron showing a spectrum characteristic of methemoglobin. See the spectra in Figure 29 of WO 93/09806, and item 6 on page 4 of the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 mailed to the United States Patent and Trademark Office on 6 January 1999.

The subject application and priority applications describe a further finding which would be unexpected to one of ordinary skill in the art at the time of the invention. At that time, the object was to make a form of oxygen-carrying hemoglobin. Although SNO-hemoglobin in the

deoxy and met forms are vasodilators, as would be expected of NO donors, the oxy form of SNO-hemoglobin, when administered alone, can act as a vasoconstrictor. See in the written description Example 4, especially page 57, lines 3-14, and Figure 4A.

Nitrosylhemoglobin was only briefly mentioned in the Stamler *et al.* WO 93/09806 reference, on page 58, line 21. In WO 93/09806, and in all other prior art known to Applicants, nitrosylhemoglobin was never discussed as being, or possibly being, a donor of NO, nor is any reaction described by which nitrosylhemoglobin can be converted to S-nitrosohemoglobin or anything else that could act as a donor of NO. Polynitrosated hemoglobin species were not discussed at all in WO 93/09806.

INTERVIEW SUMMARY

The subject application and its immediate continuation-in-part application, 08/874,992, were discussed in a telephonic interview with Examiner Celsa on February 13, 2001, with Applicant Jonathan S. Stamler and the undersigned attorney, Carol A. Egner, participating. A summary of the discussion with respect to the subject application appears below.

The terms defining the various forms of hemoglobin and what each form is were discussed. In particular, the scope of the term "nitrosated hemoglobin" was discussed. The definition for this term appearing on page 27, lines 9-20, was pointed out, and is further elaborated upon elsewhere in this paper.

Relevant teachings about the forms of hemoglobin known in the prior art were summarized for the Examiner. Nitrosylhemoglobin was well known and characterized in the prior art, as can be seen, for example, by two references cited in the Office Action of July 31, 2000, Moore *et al., J. Biol. Chem. 251*:2788-2794 and Sharma *et al., J. Biol. Chem. 253*:6467-72. The extremely low dissociation constants for nitrosylhemoglobin given in these cited references and in other references provide an indication of the stability of the NO-Fe bond, and accordingly, the complete lack of any possible NO donor activity.

The physiological effects of administering purified, unmodified hemoglobin to a human or other mammal were brought up. Hemoglobin is known from prior art studies to act as a

scavenger of NO, effectively binding the NO irreversibly, or eliminating the NO by its conversion to nitrate. Hemoglobin produces a vasoconstrictive effect, and activates platelets in the blood clotting process. In some cases, the effects of administering hemoglobin can be fatal.

The new findings described in the patent application and the inventions arising from them were summarized for the Examiner. The properties of SNO-hemoglobins, which can be used as NO donors, and the properties of nitrosylhemoglobin, which can be converted *in vivo* to SNO-hemoglobin and can produce the same physiological effects, were described. These properties are further developed elsewhere in this paper.

The Examiner stated that in Claim 16, the meaning of "regulating" should be clarified. Claim 16 has been amended to clarify its meaning. It was explained to the Examiner that Claim 16 arises from the phenomenon of transnitrosation, the transfer of biological equivalents of NO from one molecule to another. For example, NO can be transferred from SNO-hemoglobin to thiol to produce an S-nitrosothiol, and from the S-nitrosothiol to a biologically active form of NO in tissues.

The teachings of the Wade and Castro reference were discussed (Wade and Castro, *Chem. Res. Tox. 3*:289-291, 1990). It was presented to the Examiner that the reaction described in this paper included as reactants not only hemoglobin and NO, but also a small organic nucleophile. The products were the nitrosylated small organic nucleophile (phenol nitrosylated at O, acetylcysteine nitrosylated at N, or proline nitrosylated at N) and nitrosylhemoglobin, with NO bound at the heme Fe. NO was not reported to be, or speculated to be, at any other site in hemoglobin.

Based on the Declaration of Jonathan S. Stamler, M.D. Under 37 C.F.R. § 1.132 filed on 6 January 1999 and exhibits accompanying the Declaration, the Examiner stated that he has accepted that WO 93/09806 does not present an enabling description of a method to produce S-nitrosohemoglobin. There remained a question of whether other species of nitrosated hemoglobin could have been produced from methods described in WO 93/09806. It was presented to the Examiner that it would be very unlikely that NO adducts were produced at O, N or C atoms in the hemoglobin molecule, if none could be detected at S atoms, because the thiol

groups were the most reactive nucleophilic sites. The product of the method used on hemoglobin was oxidized at the heme (methemoglobin). WO 93/09806 does not present a method to produce nitrosylhemoglobin, and before the invention by Applicants, nitrosylhemoglobin would not have been expected to be, or to be converted to, a donor of NO.

It was emphasized that hemoglobin is very unlike the other proteins described in WO 93/09806 and other proteins that do not have hemes, because of its property of binding NO at the heme Fe. It was also emphasized that once SNO-hemoglobin was produced by Applicants, it was not in all cases a vasodilator. In the form desired to be used for enhanced oxygen delivery, as expressed in WO 93/09806, SNO-hemoglobin acted as a vasoconstrictor (SNO-oxyHb). One of ordinary skill in the art could not have predicted this effect from the teachings of WO 93/09806.

CONCLUSION

Applicants believe that with the amendments made herein, the claims are in allowable condition. The Examiner is respectfully requested to consider the above amendments and remarks, and to reconsider the application. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

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Dated: April 12, 2001

MARKED UP VERSION OF AMENDMENTS

Specification Amendments Under 37 C.F.R. § 1.121(b)(1)(iii)

Replace the paragraph at page 76, line 28 through page 77, line 29 with the below paragraph marked up by way of bracketing and underlining to show the changes relative to the previous version of the paragraph.

We proposed that the degree of hydrogen bonding between bound oxygen and the distal histidine was critical in determining the degree of oxidation of hemoglobin by nitric oxide. Therefore, we examined the degree of oxidation of hemoglobin by nitric oxide in a variety of buffers. 5 ml of phosphate buffer containing 300 μM Hemoglobin A (~95% oxyHb) was placed in a 15 ml vial. Nitric oxide was added from a stock solution, 2 mM, stored under nitrogen. Immediately after nitric oxide addition, the absorbance at 630 nm was measured, and the concentration of oxidized (metHb) was plotted, using 4.4 as the extinction coefficient for metHb at 630 nm. Experiments were performed in 1 M, 100 mM, and 10 mM sodium phosphate buffer (pH 7.4). The data in Figure 19 show higher oxidized hemoglobin formation in 1M phosphate, which is indicative of a higher effective substrate concentration, as would be predicted by phosphate destabilization of the hydrogen bond between iron bound oxygen and the distal histidine. At the lowest concentrations of nitric oxide added, S-nitrosothiol was formed under all conditions (approximately 5 μM). Additions of nitric oxide at concentrations of 30 μM or greater resulted in the additional formation of nitrite. The presence of 200 mM borate within the buffer reduced oxidized hemoglobin and nitrite formation, whilst the presence of either 200 mM <u>acetate</u> or chloride increased the formation of oxidized hemoglobin and nitrite. Addition of nitric oxide to hemoglobin in 10 mM phosphate buffer at a ratio of less than 1:30 (NO:Hemoglobin A) resulted in the formation of S-nitrosothiol without production of oxidized hemoglobin. S-nitrosothiol formation was optimized by adding the nitric oxide to hemoglobin in 10 mM phosphate, 200 mM borate, pH 7.4. Therefore, the balance between oxidation and nitrosothiol formation is dependent upon the ratio of nitric oxide to hemoglobin and the buffer environment.

Claim Amendments Under 37 C.F.R. § 1.121(c)(1)(ii)

16. (Twice Amended) A method for [regulating] potentiating delivery of [oxygen and] NO to tissues in a mammal, comprising administering to the mammal an effective amount of a mixture of a low molecular weight thiol [or nitrosothiol] and hemoglobin or nitrosated hemoglobin.

67. (Amended) A composition [consisting essentially of] comprising S-nitrosylated oxyhemoglobin [without detectable oxidation of the heme].

68. (Amended) A method for making a composition [consisting essentially of] comprising S-nitrosylated oxyhemoglobin [without detectable oxidation of the heme], comprising incubating excess nitrosating agent with purified hemoglobin in the presence of oxygen at pH 7.4 to 9.2.

70. (Amended) A composition [consisting essentially of] comprising S-nitrosylated deoxyhemoglobin [without detectable oxidation of the heme].

71. (Amended) A method for making a composition [consisting essentially of] comprising S-nitrosylated deoxyhemoglobin, comprising incubating excess nitrosating agent with purified hemoglobin in the absence of oxygen at pH 7.4 to 9.2.